Methyl Esterification of Waste Fatty Acids with Immobilized Candida antarctica Lipase

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Abstract: Enzymatic production of a docosahexaenoic acid-rich oil from tuna oil creates considerable amounts of free fatty acids (FFA), which are treated as an industrial waste but the handling is difficult because of their solid state. We thus attempted to convert the waste FFA (Tuna-FFA) to their methyl esters through an organic solvent-free enzymatic process. When waste Tuna-FFA were esterified at 30°C with two molar equivalents of methanol (MeOH) using 1.0 wt% immobilized Candida antarctica lipase, the esterification degree reached 95% after 24 h. The reaction was recycled by transferring the enzyme into a fresh substrate mixture every 24 h, but the esterification degree maintained 95% during 45 cycles. To further increase the esterification degree, the esterification was repeated with 5 molar equivalents of MeOH against the remaining FFA after dehydration of the mixture obtained by the single reaction. The repeated reaction increased the esterification degree to 97-98%.

Key words: Candida antarctica, lipase, immobilized enzyme, methyl esterification, waste fatty acids

1 Introduction

Polyunsaturated fatty acid (PUFA)-rich oils are produced by selective hydrolyses of natural PUFA-containing oils with a lipase acting on PUFA very weakly (1-3), and are used as nutraceutical lipids. An oil containing 50% docosahexaenoic acid (22:6n-3; DHA) has been industrially produced by hydrolyzing tuna oil at ca. 75% using Candida rugosa lipase (1). An oil containing 45% γ-linoleic acid (18:3n-6) has also been produced similarly by hydrolyzing borage oil at ca. 60% (3,4). Considerable amounts of free fatty acids (FFA) created by the enzymatic process are treated as industrial wastes, but the handling is difficult because they are in solid state at ambient temperature. Meanwhile, methyl esterification of the FFA decreases their melting point, and the resulting esters may be used as biodiesel fuel. FFA easily undergo methyl esterification by a chemical process with several molar equivalents of methanol (MeOH) and an acid catalyst, e.g. sulfuric acid, but the catalyst should be removed to make use of the product as a fuel. The removal by neutralization...
with alkali is not favorable because fatty acid alkali salts (soaps) contamine the product. The removal should therefore be conducted by washing with a large amount of water, which creates other environmental concerns. These problems can be solved by adopting enzymatic process.

There have been few reports on esterification of FFA with MeOH or ethanol (EtOH) (5-8), although S. Bloomer et al. achieved an efficient organic solvent system of producing long-chain fatty acid ethyl esters with immobilized Rhizomucor miehei lipase (6,7). The application of their system to conversion of waste FFA to their methyl esters, however, requires the removal of solvent after the reaction. Meanwhile, because we recently developed an effective process of ethyl esterification of DHA in an organic solvent-free system (8), the process was applied to methyl esterification of waste FFA (referred to as waste Tuna-FFA) which were discharged from the industrial enzymatic hydrolysis of tuna oil for producing DHA-rich oil. In this paper, we describe a process in which waste Tuna-FFA are successfully converted to their methyl esters in an organic solvent-free system.

2 Materials and Methods

2.1 Materials

Waste Tuna-FFA were provided by Maruha Corp., Tokyo. The FFA contained 290 ppm water, and was composed of 3.1 wt% myristic acid (14:0), 18.4 wt% palmitic acid (16:0), 5.2 wt% palmitoleic acid (16:1n-7), 4.7 wt% stearic acid (18:0), 22.8 wt% oleic acid (18:1n-9), 3.8 wt% arachidonic acid (20:4n-6), 7.0 wt% eicosapentaenoic acid (20:5n-3), and 14.4 wt% DHA. The acid value was 197 mg KOH/g (3.51 mmol/g), and the peroxide value was 8.2 milliequivalents/kg. MeOH, n-propanol, lauric acid were purchased from Wako Pure Chemical Industry Ltd., Osaka. Immobilized Candida antarctica lipase (Novozym 435) was a gift from Novozymes (Bagsvaerd, Denmark). Other chemicals were of analytical grade.

2.2 Reactions

One-step methyl esterification of waste Tuna-FFA was carried out at 30°C in a screw-capped vessel with two molar equivalents of MeOH using 1.0% immobilized C. antarctica lipase by weight of the reaction mixture, with shaking at 130 oscillations/min. To increase the esterification degree, two-step reaction was performed. The first-step reaction was performed in a mixture of waste Tuna-FFA, one or two molar equivalents of MeOH, and 1.0 wt% C. antarctica lipase. The second-step reaction was conducted with 5 molar equivalents of MeOH against the remaining FFA after dehydration of mixture obtained by the first-step reaction. The reaction was recycled by transferring the enzyme to a fresh substrate mixture every 24 h. Esterification of lauric acid was similarly performed with two molar equivalents of n-propanol using 0.3 wt% immobilized C. antarctica lipase. The esterification degree was calculated based on the acid values of the reaction mixture before and after the incubation, which were determined by titration with KOH solution.

2.3 Analyses

FFA were methylated in 3 mL of 5% HCl-MeOH by heating at 70°C for 10 min. Fatty acid methyl esters (FAME) were analyzed with a Hewlett-Packard 5890 gas chromatograph (Avondale, PA, USA) connected to a DB-23 capillary column (0.25 mm × 30 m; J & W Scientific, Folsom, CA, USA) as described previously (9). The weight contents of FFA and FAME were analyzed by a thin-layer chromatograph/flame-ionized detector analyzer (Iatroscan MK-5; Iatron Laboratories Inc., Tokyo) after development with a mixture of n-hexane:ethyl acetate:acetic acid (90:10:1, by volume). Water content was analyzed by Karl Fisher titration (Moisture Meter CA-07; Mitsubishi Chemical Corp., Tokyo). Kinematic viscosity, specific gravity, flash point, and cetane index of the reaction product was determined according to the International Organization for Standardization (ISO) method of ISO3104, ISO3675, ISO2719, and American Society for Testing and Material (ASTM) D 976, respectively.

3 Results

In general, lipases weakly act on PUFA, especially on DHA. But C. antarctica lipase was known to recognize well not only C18 FFA but DHA as substrates (8, 10-12). In addition, the lipase strongly acted on short-chain alcohols, MeOH and EtOH (8, 12-14). Because waste Tuna-FFA included ca. 30 wt% PUFA, we selected immobilized C. antarctica lipase for their methyl esterification.
3.1 Effect of Enzyme Amount on Methyl Esterification of Waste Tuna-FFA

Waste Tuna-FFA were esterified at 30°C with two molar equivalents of MeOH using various amounts of immobilized C. antarctica lipase. Figure 1 shows esterification degree after 1, 3, and 24 h. The initial reaction velocity shown by 1- and 3-h esterification depended on the amount of lipase, and the esterification degree after 24 h reached 95% using >0.5 wt% immobilized lipase.

3.2 Effect of MeOH Amount

Waste Tuna-FFA were esterified with various amounts of MeOH using 0.5 wt% C. antarctica lipase (Fig. 2). Initial reaction velocity shown by esterification degrees after 1 and 3 h decreased with increasing the amount of MeOH, showing that excess amounts of MeOH inhibited the esterification. Esterification degree after 24 h was 84.3% in the reaction with an equimolar amount of MeOH. The degree increased to 95.4% by addition of two molar equivalents of MeOH, but did not increase to >95% by further addition.

We have reported C. antarctica lipase is inactivated by a high concentration of MeOH (13,14). To investigate the lipase stability against MeOH, the reactions in Fig. 2 were repeated 5 cycles by transferring enzymes to fresh substrate mixtures every 24 h. Figure 3 shows the relative initial velocity in the 1st, 3rd, and 5th cycles. The velocities in the reactions with one and two molar equivalents of MeOH decreased to 90% after 5 cycles. Further decrease was observed in the reactions with >4 molar equivalents of MeOH, showing that immobilized C. antarctica lipase was unstable in the presence of larger amounts of MeOH.

3.3 Effect of Reaction Temperature

A mixture of waste Tuna-FFA/MeOH (1:2, mol/mol) was shaken with 0.5 wt% immobilized C. antarctica lipase at temperatures in the range from 20°C to 50°C. The initial velocity depended on the temperature: the esterification degrees after 1 h at 20, 30, 40, and 50°C were 29.6, 35.2, 39.2, and 44.7%, respectively. This result showed that the optimum temperature was >50°C.

Repeated use of immobilized lipase may decrease the activity resulting from the effects of MeOH and temperature. Hence, the methyl esterification of waste Tuna-FFA was recycled by transferring the lipase to a fresh substrate mixture every 24 h. In addition, a reaction for measuring esterification activity was repeated as a control without MeOH, which was performed at 40 and 50°C in a mixture of lauric acid/n-propanol (1:2, mol/mol) and 0.3 wt% immobilized lipase. The control reaction was not performed at <30°C because the substrate mixture did not melt at these temperatures. Fig.
Figure 4 shows the relative initial velocity in each cycle. The activities of methyl esterification of waste Tuna-FFA at 20, 30, and 40°C after 10 cycles decreased to 83, 84, and 61% of those in the first cycle, respectively, and the activity after 4 cycles at 50°C decreased to 35% of that in the first cycle. On the other hand, propyl esterification of lauric acid at 40 and 50°C after 10 cycles decreased to 92 and 83% of those in the first cycle, respectively. These findings showed that *C. antarctica* lipase inactivated a little in the presence of two molar equivalents of MeOH against FFA, and that the inactivation was accelerated at >40°C.

Based on studies of several factors affecting the methylation of waste Tuna-FFA, the conditions for production of FAME by a single reaction were decided as follows: the molar ratio of MeOH to FFA, 2:1; reaction temperature, 30°C; reaction period, 24 h. The amount of immobilized lipase was fixed at 1.0 wt% after considering that the enzyme is reused for long period.

3.4 Effect of Water Generated by Methyl Esterification

Single reaction with two molar equivalents of MeOH achieved 95% esterification, even though the generated water (4.9%) was not removed. The esterification degree may further be increased by removing the generated water. Hence, the product obtained from single reaction was dehydrated and then used as a substrate for the repeated esterification.

The first reaction was conducted at 30°C for 24 h with an equimolar amount of MeOH using 1.0 wt% immobilized lipase (esterification degree, 82.5%). The reaction mixture was separated to oil and water layers by allowing to stand. The oil layer (water content, 3980 ppm) was esterified at 30°C with 1, 5, and 10 molar...
equivalents of MeOH against the remaining FFA using 1.0 wt% immobilized lipase. These reactions reached steady state after 5 h, and their esterification degrees were 43.1, 64.7, and 68.2%, respectively. On the other hand, the oil layer was dehydrated at 50°C and 3 mm Hg for 1 h, and the resulting oil layer (water content, 150 ppm) was then esterified with 1, 5, and 10 molar equivalents of MeOH using 1.0 wt% immobilized lipase (Table 1). Esterification degrees in 5-h reactions with 1 and 5 molar equivalents of MeOH were 53.8 and 82.1%, respectively. Though 10 molar equivalents of MeOH was used in the second-step reaction, the esterification degree did not increase. In the reaction with 5 molar equivalents of MeOH, 96.7% of Tuna-FFA were converted to their methyl esters in total. These results indicated that removal of generated water increases the esterification degree to nearly 97%.

When waste Tuna-FFA was esterified at 30°C for 24 h with two molar equivalents of MeOH using 1.0 wt% immobilized lipase, the esterification degree reached 95.2%. The dehydrated reaction mixture (water content, 145 ppm) was also esterified at 30°C with 1, 5, and 10 molar equivalents of MeOH against the remaining FFA using 1.0 wt% immobilized lipase (Table 1). The reactions reached steady state after 5 h. Esterification degrees in the reactions with 1 and 5 molar equivalents of MeOH were 26.5 and 52.0%, respectively. When 5 molar equivalents of MeOH was used in the second-step reaction, 97.7% of Tuna-FFA were converted to their methyl esters. The conversion was only 1% higher than that in the reaction with an equal amount of MeOH in the first-step reaction. These results may suggest that the economical amounts of MeOH in the first- and second-step reactions are 1 and 5 molar equivalents against FFA, respectively.

### 3.5 Lipase Stability in Continual Batch Methyl Esterification of Waste Tuna-FFA

Single reaction was performed in a mixture of waste Tuna-FFA/MeOH (1:2, mol/mol) and 1.0 wt% immobilized *C. antarctica* lipase. To investigate the stability of the lipase preparation, the reaction was recycled by transferring the enzyme to a fresh substrate mixture every 24 h. As shown in Fig. 5, esterification degree after 1 h decreased from 51.3 to 20.5% during 45 cycles (half-life of the preparation, 35 d), although the degree after 24 h maintained 95% because an excess amount of lipase was used as a catalyst.

Two-step methyl esterification of waste Tuna-FFA was also recycled using 1.0 wt% of the lipase (Fig. 5). The first-step reaction was recycled for 45 cycles with an equimolar amount of MeOH. The reaction mixture of each cycle was gathered and then dehydrated. The resulting mixture contained 84.6% FAME, and the water content was 95 ppm. The second-step reaction was conducted by adding 5 molar equivalents of MeOH against the remaining FFA. Esterification degree after 1 h in the first-step reaction decreased from 65.0 to 29.2% during 45 cycles, and the degree in the second-

| Table 1 Two-Step Methyl Esterification of Waste Tuna-FFA with *C. antarctica* Lipase. |  |
|---|---|---|
| **First reaction**<sup>a</sup> | **Second reaction**<sup>b</sup> | **Total esterification** |
| FFA/MeOH (mol/mol) | Esterification (%) | FFA/MeOH (mol/mol) | Esterification (%) | |
| 1:1 | 82.5 | 1:1 | 53.8 | 91.9 |
| 1:2 | 95.2 | 1:5 | 82.1 | 96.9 |
| 1:10 | 81.4 | 96.7 |
| 1:1 | 26.5 | 96.4 |
| 1:5 | 52.0 | 97.7 |
| 1:10 | 54.8 | 97.8 |

<sup>a</sup> A 80-g mixture of waste Tuna-FFA and MeOH was shaken at 30°C for 24 h with 1.0 wt% immobilized *C. antarctica* lipase.

<sup>b</sup> The first reaction mixture was separated to oil and water layers, and the oil layer was dehydrated at 50°C and 3 mm Hg for 1 h. A 10-g mixture of the dehydrated oil layer and 1, 5, and 10 molar equivalents of MeOH was shaken at 30°C for 5 h with 1.0 wt% immobilized *C. antarctica* lipase.
step reaction decreased from 47.7 to 20.7% during 40 cycles. These results showed that the enzyme activity fell to half of the initial value after 35 d in the first- and second-step reactions. Esterification degrees after 24 h in the first- and second-reactions, however, maintained 85 and 82% during 40 d, respectively.

3.6 Some Properties of Reaction Product
All of the mixture recovered from the two-step reaction in Fig. 5 was dehydrated at 70°C and 3 mm Hg for 1 h. The FAME content in the resulting product was 97.1%. Fatty acid composition in the methyl ester fraction was as follows: 3.2 wt% myristic acid, 19.3 wt% palmitic acid, 5.2 wt% palmitoleic acid, 4.5 wt% stearic acid, 23.5 wt% oleic acid, 3.3 wt% arachidonic acid, 7.1 wt% eicosapentaenoic acid, and 13.8 wt% DHA. The composition agreed with that of waste Tuna-FFA, showing that C. antarctica lipase acts on PUFA as strongly as on C14-18 fatty acids.

Some fuel properties measured according to the method of ISO were as follows: kinematic viscosity at 30°C, 7.1 mm²/s; specific gravity, 0.891 (15°C/4°C); flash point, >110°C; and cetane index, 50.5. Acid value of the product was high (5.9 mg KOH/g), but the product did not contain any fatty acid alkaline salts (soaps). Hence, the product may be used as a biodiesel fuel.

4 Discussion
We have described an efficient process for methyl esterification of waste Tuna-FFA with immobilized C. antarctica lipase. The process has the following features: (i) single reaction with two molar equivalents of MeOH achieves 95% esterification, and two-step reaction including removal of generated water attains to 97% esterification; (ii) large amounts of MeOH are not required (>95% esterification can be obtained using about two molar equivalents of MeOH against FFA); (iii) the lipase catalyst can be used for >40 d, leading to the reduction in the production cost; (iv) any organic solvents are not necessary; (v) the product (oil layer) is obtained only by allowing to stand; (vi) the product may be used as biodiesel fuel because it is not contaminated with acid and soaps.

In methanalysis of triacylglycerols, >1/2 molar equivalent of MeOH for the stoichiometric amount inactivated C. antarctica lipase irreversibly (13). The solubility of MeOH is 1/2 of the stoichiometric amount. We thus hypothesized that the irreversible inactivation of the lipase is due to the contact of the enzyme molecule with insoluble MeOH, which exists as drops in the reaction mixture. Actually, the lipase did not inactivate in the presence of 1/3 molar equivalent of MeOH for the stoichiometric amount, and 95% triacylglycerols were efficiently converted to their methyl esters by the stepwise addition of MeOH. In this study, the substrates are FFA and MeOH, and the two substances dissolve each other. As MeOH existed in a soluble state, significant inactivation was not observed.
even in the presence of 10 molar equivalents of MeOH against FFA (MeOH content, 53 wt%) (Fig. 4). These facts indicate that a reaction system, in which MeOH is in a soluble state, should be constructed to get a high efficiency when MeOH is used as one of the substrates.

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